European Commission

ERM
European Reference Materials

CERTIFICATION REPORT

The certification of different mass fractions of DAS-44406-6 in soya seed powder: ERM®-BF436a, ERM®-BF436b, ERM®-BF436c, ERM®-BF436d and ERM®-BF436e

Certified Reference Materials
ERM®-BF436a, ERM®-BF436b, ERM®-BF436c,
ERM®-BF436d and ERM®-BF436e

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ERM®-BF436a, ERM®-BF436b, ERM®-BF436c,
ERM®-BF436d and ERM®-BF436e

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Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the production of a set of Certified Reference Materials (CRMs) ERM-BF436a, b, c, d and e, certified for their DAS-44406-6 mass fractions. The material was produced following ISO Guide 34:2009 [1].

Genetically modified (GM) seeds of the soya event DAS-44406-6 and of a non-GM soya variety were milled to obtain GM and non-GM powders. Gravimetric mixtures of non-GM and GM soya powder were prepared by dry-mixing.

Between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2].

The certified value was obtained from the gravimetric preparations, taking into account the purity of the base materials and their water mass fraction. The certified values were confirmed by event-specific real-time PCR as independent verification method (measurements within the scope of accreditation to ISO/IEC 17025:2005 [3]).

Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).

The materials are intended for the calibration or quality control of methods. As any reference material, they can be used for establishing control charts or validation studies. The CRMs are available in glass vials containing at least 1 g of dried soya seed powder and closed under argon atmosphere. The minimum amount of sample to be used is 200 mg.

The CRMs were accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

The following values were assigned:

	DAS-44406-6 mass fraction [1)]	
	Certified value [2)] [g/kg]	Uncertainty [3)] [g/kg]
ERM-BF436a	< 0.06	-
ERM-BF436b	> 986	-
ERM-BF436c	1.00	0.14
ERM-BF436d	10.0	1.0
ERM-BF436e	100	9

1) Genetically modified soya event with the unique identifier DAS-444Ø6-6.

2) Mass fraction of DAS-44406-6 soya based on the masses of genetically modified DAS-44406-6 soya seed powder and non-modified soya seed powder and their respective water content. The certified values and their uncertainties are traceable to the International System of units (SI).

3) The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

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Glossary

2mEPSPS	Double mutant 5-enolpyruvylshikimate-3-phosphate synthase
AAD-12	Aryloxyalkanoate dioxygenase-12
ANOVA	Analysis of variance
b	Slope in the equation of linear regression y = a + *b*x
Cq	Quantification cycle (also referred to as threshold cycle, Ct)
CRM	Certified reference material
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EC	European Commission
ERM®	Trademark of European Reference Materials
EU	European Union
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
GM	Genetically modified
GMO	Genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurements *[ISO/IEC Guide 98-3:2008]*
EDTA	Ethylenediamintetraacetic acid
IEC	International Electrotechnical Commission
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization
JRC	Joint Research Centre
k	Coverage factor
LOD	Limit of detection
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not applicable
n.c.	Not calculated
PAT	Phosphinothricin-N-acetyltransferase
PCR	Polymerase chain reaction
PSA	Particle size analysis
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
rpm	Revolutions per minute
RSD	Relative standard deviation
RSE	Relative standard error (= RSD/$\sqrt{n}$)
RT	Room temperature
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation; an additional index "rel" is added as appropriate
t	Time
t_i	Time point for each replicate
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation in real-time PCR
TE	Tris-EDTA
u	Standard uncertainty
U	Expanded uncertainty

u^*_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by the intermediate precision of the method; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
VIM	International Vocabulary of Metrology – Basic and General Concepts and Associated Terms *[ISO/IEC Guide 99:2007]*
V-KFT	Volumetric Karl Fischer titration
$\overline{x}$	Arithmetic mean
$\overline{y}$	Mean of all results of the homogeneity study
ν	Degrees of freedom

1 Introduction

1.1 Background: need for the CRM

Legislation in the EU regulates the placing on the market of food and feed consisting of, containing or produced from genetically modified organisms (GMOs). They are referred to as 'GM food and feed' and require authorisation before being placed on the market in the EU. Food and feed products containing materials which contain, consist of or are produced from GMOs in a proportion higher than 0.9 percent of the food and feed ingredient considered individually or food or feed consisting of a single ingredient, need to be labelled [5]. This labelling threshold is applicable for adventitious presence of GMOs, while GMOs added on purpose need to be labelled independent from a threshold. Additionally feed may contain 0.1 mass percent of a GM event for which an authorisation process is pending or the authorisation in the EU has expired [6]. These thresholds demand on the one hand the development and validation of reliable methods for GMO quantification, and on the other hand the production of reference materials for calibration or quality control of these methods.

Dow AgroSciences (Oxon, UK) has developed the genetically modified (GM) soya event DAS-444Ø6-6 (unique identifier code following Commission Regulation (EC) No 65/2004 [7], further referred to as DAS-44406-6) and has asked in 2012 the Institute for Reference Materials and Measurements (IRMM, Geel, BE) to produce a reference material for the quantification of DAS-44406-6 soya. The event DAS-44406-6 expresses the AAD-12, PAT and 2mEPSPS proteins in soya, derived from *Delftia acidovorans*, *Streptomyces viridochromogenes* and maize, providing tolerance to application of aryloxyalkanoate herbicides (such as 2,4-D), glufosinate and glyphosate, respectively [8]. The Certified Reference Material (CRM) produced by IRMM has been named ERM-BF436 and is composed of a set of five CRMs with different mass fractions of DAS-44406-6 soya.

1.2 Choice of the material

The set of CRMs ERM-BF436 was produced from milled GM seeds and non-GM seeds. Seeds were selected as source for the raw material due to their high purity, compared to harvest materials.

1.3 Design of the project

Besides the pure non-GM material ERM-BF436a and the pure GM material ERM-BF436b, all gravimetric mixtures of non-GM and GM soya powder were prepared by dry-mixing. The first material ERM-BF436e was prepared by mixing pure GM with non-GM soya powder. ERM-BF436d was prepared by further dilution of ERM-BF436e and ERM-BF436c was prepared by further dilution of ERM-BF436d, in both cases with non-GM soya powder.

The different mass fractions of ERM-BF436 were certified using a gravimetric approach and details are described in this certification report.

2 Participants

2.1 Raw material provider

Dow AgroSciences, Oxon, UK

2.2 Project management, processing, characterisation and certification

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM, measurements under the scope of ISO/IEC 17025 accreditation BELAC No. 268-TEST)

3 Material processing and process control

3.1 Origin of the starting material

For the preparation of the candidate CRMs, Dow AgroSciences supplied non-GM soya seeds and DAS-44406-6 soya seeds to IRMM. After arrival, the soya seeds were stored at (4 ± 3) °C in the dark until used for processing. The purities of the delivered non-GM and GM soya seeds were investigated at IRMM as follows.

For the GM soya seeds, the purity was measured by analysing 219 randomly selected GM seeds for the presence of the GM event DAS-44406-6. In order to get rid of any possible contamination attached to the seeds, they were planted and genomic DNA was extracted from the seedling's leaves using the DNeasy Plant Mini kit (Qiagen, Venlo, NL). To identify the seeds with respect to the DAS-44406-6 event, quantitative real-time PCR was then performed according to the event-specific real-time PCR method delivered under confidentiality agreement to IRMM. This method will be published after completion of its international validation on the homepage of the European Reference Laboratory for GM food and feed (EURL-GMFF) [9]. Genomic DNA extracted from pure DAS-44406-6 soya powder was used as positive control. Detection was done on an ABI 7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [10]. The results showed that all plants from the GM seed batch gave a signal for presence of the DAS-44406-6 event. Statistical analysis of the 219 measurements (Poisson distribution for rare events) revealed that the GM soya seed batch had a purity of > 98.6 % (95 % level of confidence). The calculated lot purity of the GM seed batch was taken into account for the estimation of the uncertainties associated to the certified values of the CRMs (Section 6.2).

The purity of the non-GM seed batch was investigated on the processed powder. Real-time PCR measurements on the non-GM soya seed powder were performed with a limit of detection (LOD) of 0.06 g/kg. The method did not detect the event DAS-44406-6 (Section 3.5). The LOD of the method was taken into account for calculating the certified value of ERM-BF436a (Section 7).

3.2 Processing

All soya seeds received by IRMM were rinsed with water, drained, and dried on special trays in a drying chamber of a freeze-dryer at 30 °C for 20 h (Epsilon 2-65D, Osterode, DE). The mass fraction of water was determined by volumetric Karl Fischer titration (V-KFT). After the washing and drying step, the non-GM seeds had a remaining residual water mass fraction of about 50 g/kg and the GM seeds had a remaining residual water mass fraction of about 40 g/kg.

About 23 kg of non-GM soya seeds and 10 kg of DAS-44406-6 soya seeds were used for the processing of ERM-BF436. The GM and non-GM base materials were processed separately. Cross-contamination and contamination with foreign DNA were avoided applying systematic cleaning, clean laboratory clothing and measures to prevent cross-contamination by air. All contact surfaces were treated with a DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) prior to exposure to the materials. An in-house validation

study had proven beforehand that the solution degraded DNA effectively under the given conditions. If required, the base powders were stored for short time periods in closed plastic containers.

The seeds were milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). Prior to this milling step, the soya seeds were frozen overnight in approximately 6 kg portions in stainless steel containers immersed in liquid nitrogen. Additionally, the mill was cooled to process the seeds at a temperature below -90 °C. The feeding speed of the mill was adjusted to ensure most efficient milling with respect to the particle size obtained. After milling, the powder was kept at -20 °C. The GM and non-GM powders were then sieved separately with a 500 µm stainless steel mesh on a sieving machine (Russel Finex, London, UK). In case of the GM powder, a coarse fraction of 78 g did not pass the 500 µm mesh and was discarded. For the non-GM powder a coarse fraction of 135 g did not pass the 500 µm mesh and was discarded. The remaining powder of each base material, which passed the sieve, was mixed in a DynaMIX CM200 (WAB, Basel, CH) for 1 h to improve equal distribution of the different types of soya tissues because the milling and sieving processes applied foster the separation of the different tissues from each other.

For the non-GM and GM powders, a residual water mass fraction of (69.9 ± 9.9) g/kg and (57.1 ± 8.1) g/kg respectively was measured by volumetric Karl Fischer titration (758 KFD Titrino, Metrohm, Herisau, CH) with the expanded uncertainty calculated with a coverage factor of $k = 2$. In order to facilitate the dry mixing, the water content was reduced. The powders were dried overnight under vacuum in a freeze-dryer (Epsilon 2-65D, Osterode, DE) at 30 °C. The final water mass fractions ($N = 1$, $n = 3$) of the non-GM powder and the GM powder were (9.0 ± 1.3) g/kg (U, $k = 2$) and (9.3 ± 1.3) g/kg (U, $k = 2$), respectively. The particle volumes for both powders were measured based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE) and compared (Figure 1). The mean particle diameters ($N = 1$, $n = 5$), calculated by the PSA software, were 99 µm ($s = 5$ µm) for the non-GM powder and 93 µm ($s = 9$ µm) for the GM powder. It is important to understand that the cumulative volume distribution of particles derived from laser light scattering data is based on their equivalent spherical diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles. Since most particles are presumably not perfectly spherical, the calculated volume of the particles based on their diameter is, therefore, overestimating the mean particle size. It has been concluded that the particle volume fractions of the non-GM and GM base powders were sufficiently similar to allow the processing of mixtures without introducing a bias based on the DNA extractability.

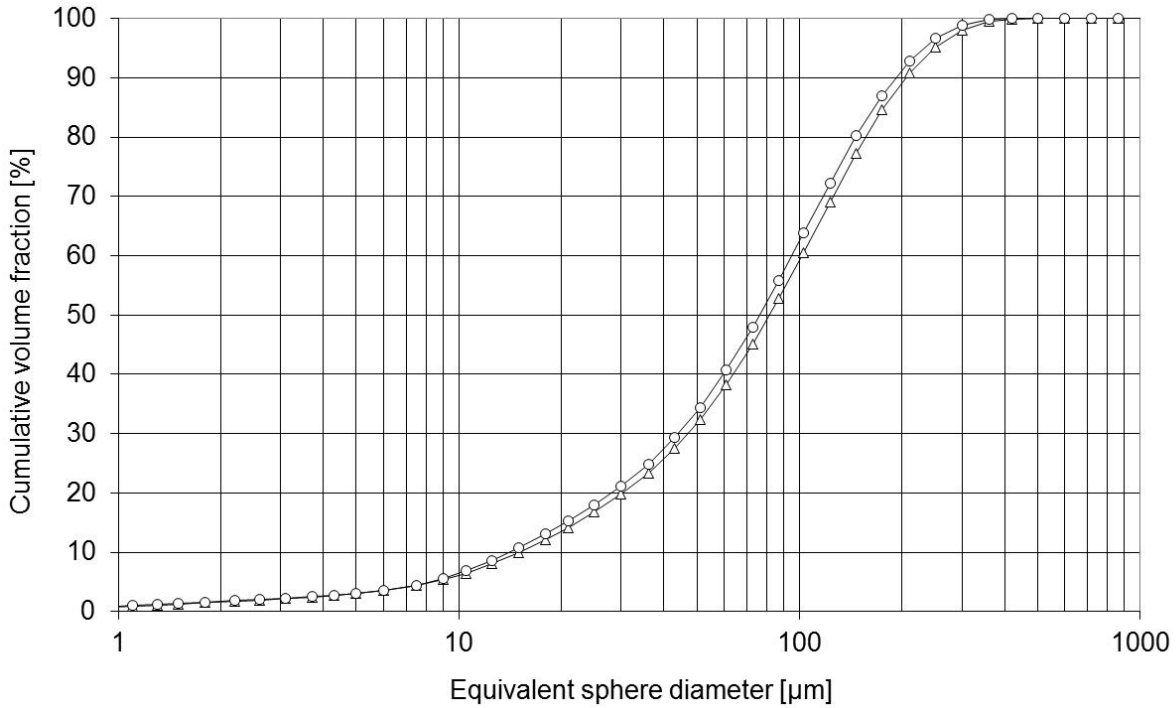

Figure 1: Cumulative representation of particle volume fractions in the GM powder (○) and non-GM powder (△) analysed by PSA (N = 1, n = 5). The total volume is set as 100 %.

The milled base materials were used to produce a blank material for DAS-44406-6 (non-GM seed powder), a pure GM DAS-44406-6 material and three mixtures containing different mass fractions of DAS-44406-6 soya seed powder in non-GM soya seed powder at nominal mass fraction levels of 1, 10 and 100 g/kg. The term "nominal" is used to distinguish between the value targeted in the processing and the certified value assigned after completion of the certification process.

All these materials, including the blank material, were treated according to the same procedure. The powder materials were weighed using a calibrated balance with an intermediate precision, expressed as relative standard uncertainty (u_{rel}), of 0.2 g. Calibration of the balance is carried out on an annual basis by an external company accredited for ISO/IEC 17025 calibration services; additionally the performance of the balance is verified before use. Portions were weighed into a container and mixed for 30 min by DynaMIX and further homogenised in a propeller mixer for additional 2 min. The blank material was processed first, followed by the mixtures. For the preparation of the mixtures, the masses of the non-GM and GM powders were corrected for their respective water mass fractions. During the certification process, dry masses have been used to establish the certified mass fraction (Section 6.2). The material having a nominal DAS-44406-6 mass fraction of 100 g/kg was produced by mixing pure GM with pure non-GM milled base materials. The material having a nominal DAS-44406-6 mass fraction of 10 g/kg was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder and the material with a nominal mass fraction of 1 g/kg was thereafter produced by further dilution of the 10 g/kg GM powder with pure non-GM powder. At each mixing step, the water mass fraction of the mixed materials was taken into account (Table 5). The gravimetric preparation formed the basis for the calculation of the mass fraction of the powders (Section 6).

After finalisation of the mixing steps, the powders were filled in 10 mL brown glass vials using an automatic filling device. The first 30 bottles of each batch were discarded as an additional precaution against carry-over contamination. Lyophilisation inserts were automatically placed in the bottle necks. Before final closure of the vials, air was evacuated in a freeze-dryer and

replaced by argon. The vials were finally closed inside the freeze-dryer with the help of a hydraulic device and then sealed with aluminium caps to prevent accidental opening during storage and transport. Colour-coded caps were used for easy identification of the different mass fraction levels of DAS-44406-6: nominal 0 g/kg = silver (BF436a), nominal 1000 g/kg = black (BF436b), nominal 1 g/kg = yellow (BF436c), nominal 10 g/kg = red (BF436d), nominal 100 g/kg = brown (BF436e), consistent with the cap colours of previous IRMM CRMs for GMOs. Each of the vials was identified by a numbered label indicating the ERM code and the unit number. Following the inventory and the selection of vials for future analysis according to a random stratified sampling scheme, the bottles were brought to a storage room for long-term storage in the dark at (4 ± 3) °C.

3.3 Process control

The residual mass fraction of water in five randomly selected bottles from each of the powder materials was determined by V-KFT. The results are summarised in Table 1.

Table 1: Water mass fraction in candidate CRMs ERM-BF436 determined by V-KFT (N = 5, n = 2)

Candidate CRM	Water mass fraction [g/kg]	
	$\bar{x}$	U (k = 2)
ERM-BF436a	10.1	1.4
ERM-BF436b	9.9	1.4
ERM-BF436c	10.2	1.4
ERM-BF436d	10.4	1.4
ERM-BF436e	10.7	1.5

Five randomly selected bottles from each of the powder materials were analysed twice for their particle volume distribution (N = 5, n = 2) based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE). The powders have a particle diameter below 500 µm (Figure 2). The mean particle diameters (N = 5, n = 2), calculated by the PSA software, were 94 µm (s = 7 µm), 95 µm (s = 4 µm), 94 µm (s = 2 µm), 94 µm (s = 5 µm) and 93 µm (s = 3 µm) for ERM-BF436a, b, c, d and e, respectively.

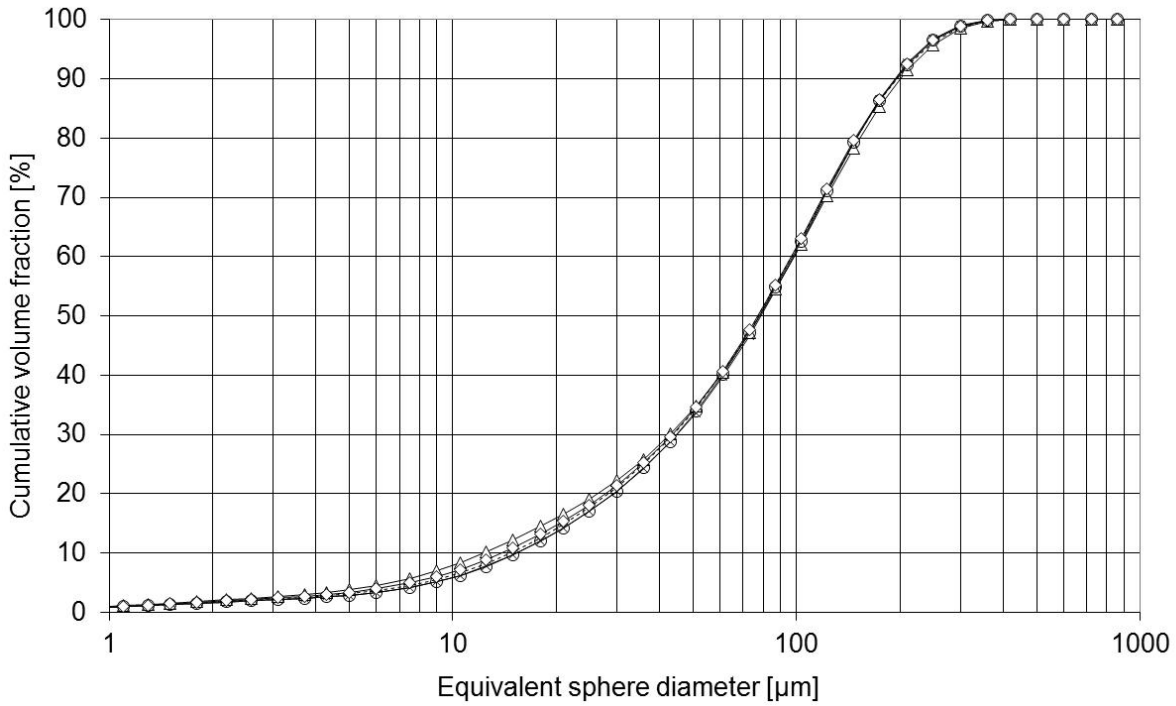

Figure 2: Cumulative representation of particle volume fractions in ERM-BF436a (x), ERM-BF436b (△), ERM-BF436c (--), ERM-BF436d (○) and ERM-BF436e (◇) analysed by PSA (N = 5, n = 2). The total volume is set as 100 %.

3.4 DNA content of the base materials

Three of the described candidate CRMs are mixtures of GM and non-GM soya seed powders, produced gravimetrically and intended to be used for calibration or quality control of quantitative measurements of the genomic DNA, following DNA extraction and purification. Consequently, any DNA mass fraction difference in the non-GM and GM base materials would lead to a shift of the measurement results obtained with e.g. real-time PCR.

The mass of DNA in both base materials was estimated using a slight modification of the classical fractionation method developed initially by Ogur and Rosen [11]. A sequential removal of alcohol-, alcohol-ether- and acid-soluble compounds and acidic extraction with 0.84 mol/L perchloric acid pH 0.3 at 70 °C was carried out. The mass of DNA was determined after derivatisation with diphenylamine using a spectrophotometer. Diphenylamine reacts specifically with 2-deoxyriboses linked to purine nucleobases [11, 12]. The extractable DNA mass fraction of the two materials was calculated as:

$$\frac{\textit{DNA mass extracted from 100 mg GM soya powder}}{\textit{DNA mass extracted from 100 mg non-GM soya powder}}$$

The ratio of the DNA mass extractable from 100 mg of GM and non-GM soya powder was found to be 1.03 ± 0.03 (N = 9 with an expanded uncertainty, k = 2). A t-test showed with 95 % confidence that no significant difference exists between the DNA mass extracted from the GM and non-GM soya powders using the modified Ogur and Rosen method.

The DNA integrity was checked by gel electrophoresis. From 200 mg samples of the processed powder materials ERM-BF436a, BF436b, BF436c, BF436d and BF436e, DNA was extracted using a CTAB DNA extraction method (Annex A). Approximately 500 ng DNA were loaded on an agarose gel (agarose mass concentration of 7.5 g/L). Staining of the DNA was done with an ethidium bromide solution (0.5 mg/L). None of the samples showed DNA degradation (data not shown).

3.5 Confirmation measurements

As a control for the gravimetric preparations, the mass fraction of DAS-44406-6 soya in the mixed materials ERM-BF436c, ERM BF436d and ERM-BF436e was confirmed by the confidential real-time PCR method provided by Dow AgroSciences targeting the transgenic DNA insertion in this soya and using a sample intake of 200 mg. The real-time PCR test was calibrated with genomic DNA extracted from the pure DAS-44406-6 soya powder. At the IRMM, the genomic DNA was extracted by an in-house validated CTAB extraction method (Annex A) using 200 mg powder samples. After the extraction, it was diluted in a TE buffer solution (pH 8.0, 1 mmol/L Tris and 0.01 mmol/L EDTA) and used to produce calibration curves for the soya-specific gene and the transgene. For the calibration curve of the soya-specific gene the DNA was used undiluted and diluted up to 200-fold. For the calibration curve of the transgene the DNA was used undiluted and diluted up to 10000-fold. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' mass fractions of DAS-44406-6 and the obtained Cq-values. The diluted DNA was used to establish the calibration points for the transgene. 3.3-fold *s* of the lowest calibration point at which RSD was below 25 % was taken to calculate the LOD of the PCR method. The results of the quantification of DAS-44406-6 are shown in Table 2. Quantification of the mass fraction of DAS-44406-6 in the powders by real time PCR confirmed the consistency of the mass fractions for the gravimetrically mixed materials ERM-BF436c, ERM-BF436d and ERM-BF436e. However, as no independent calibration was carried out, the data displayed in Table 2 can be used for confirmation of the processing, but do not necessarily resemble the true value. It has to be noted that the calibrant used for the transgenic and the soya-specific target is genomic DNA extracted from the pure DAS-44406-6 soya powder.

Table 2: Quantification of the DAS-44406-6 soya mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure DAS-44406-6 seed powder for calibration

Candidate CRM	DAS-44406-6 soya mass fraction [g/kg]	U (k = 2) [g/kg]
ERM-BF436a	< 0.06 [1) 2)]	-
ERM-BF436b	821.1 [1)]	139.6
ERM-BF436c	1.1 [3)]	0.3
ERM-BF436d	9.9 [3)]	1.6
ERM-BF436e	91.8 [1)]	14.1

1) Mean of 3 samples (extraction replicates) from each of 5 randomly selected bottles (N = 5, n = 3), with each sample measured in 3 real-time PCR replicates.

2) The obtained value is below the LOD determined during method validation (0.06 g/kg).

3) Mean of 3 samples (extraction replicates) from each of 12 randomly selected bottles (N = 12, n = 3), with each sample measured in 3 real-time PCR replicates.

4 Homogeneity

A key requirement for any reference material is the equivalence between the various units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value. In contrast to that it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 [1]

requires RM producers to quantify the between-unit variation. This aspect is covered in between-unit homogeneity studies.

This homogeneity study was planned together with the measurements to control the gravimetric preparations and the short-term stability measurements (Sections 3.5 and 5.1). As the measurement results were obtained under intermediate precision conditions on bottles randomly taken from the entire batch and analysed in a randomised order they were as well suited to investigate the homogeneity. Homogeneity of the blank material is demonstrated by the test for the purity of the raw materials (Section 3.1).

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the candidate CRMs are valid for all bottles of the material, within the stated uncertainties.

For the between-unit homogeneity test, the number of selected bottles corresponds to approximately the cubic root of the total number of the produced bottles. Therefore, 12 bottles were selected for ERM-BF436c and ERM-BF436d. In order to facilitate homogeneity studies and the short-term stability study, 15 bottles were selected for ERM-BF436e. For each candidate CRM a random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 12 and 15 groups respectively (with similar number of bottles) and one bottle was randomly selected from each group. From each bottle, 3 independent samples were taken and analysed by real-time PCR. Due to the number of PCR plates required the measurements were performed under intermediate precision conditions. Samples were analysed in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence. The results are shown in the figures in Annex B.

Regression analyses were performed to evaluate potential trends in the filling sequence. No trends were observed on a 99 % confidence level.

Furthermore, regression analyses were performed to evaluate potential trends in the analytical sequence. As this concerns an attribute of the analytical system that can be corrected for, a less stringent confidence level of 95 % was used. No statistically significant trend was detected.

The dataset was tested for consistency using single and double Grubbs outlier tests on a confidence level of 99 % on the individual results and the unit means. No outlying individual results and outlying unit means were detected.

Quantification of between-unit inhomogeneity was accomplished by analysis of variance (ANOVA), which can separate the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method intermediate precision, if the individual samples are representative for the whole bottle.

Evaluation by ANOVA requires mean values per bottle, which follow at least a unimodal distribution and results for each bottle that follow unimodal distribution with approximately the same standard deviation. Distribution of the mean values per bottle was visually tested using histograms and normal probability plots. Too few data are available for the bottle means to make a clear statement of the distribution. Therefore, it was visually checked whether the individual data follow a unimodal distribution using histograms and normal probability plots.

One has to bear in mind that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method intermediate precision, was calculated as described by Linsinger *et al.* [13]. u^*_{bb} is comparable to the LOD of an analytical

method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method intermediate precision ($s_{wb,rel}$), between–unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad \textbf{Equation 1}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \qquad \textbf{Equation 2}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}\sqrt[4]{\frac{2}{v_{MSwithin}}}}}{\bar{y}} \qquad \textbf{Equation 3}$$

MS_{within} mean of squares within-unit from an ANOVA

$MS_{between}$ mean of squares between-unit from an ANOVA

$\bar{y}$ mean of all results of the homogeneity study

n mean number of replicates per unit

$v_{MSwithin}$ degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in Table 3. In all three cases, the uncertainty contribution for homogeneity was determined by the method intermediate precision.

Table 3: Results of the homogeneity studies

Candidate CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-BF436c	18.3	6.4	5.7
ERM-BF436d	13.4	2.7	4.2
ERM-BF436e	12.4	n.c.[1)]	3.7

[1)] n.c.: cannot be calculated as $MS_{between} < MS_{within}$

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore, the between-unit standard deviation can be used as estimate of u_{bb}. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential inhomogeneity.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus can be used in an analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

Homogeneity and stability experiments were performed using a 200 mg sample intake. This sample intake gives acceptable intermediate precision, demonstrating that the within-unit inhomogeneity does no longer contribute to analytical variation at this sample intake.

5 Stability

Time, temperature and light were regarded as the most relevant influences on stability of the materials. The influence of ultraviolet or visible light was minimised by the choice of the containment which reduces light exposure. In addition, materials are stored and dispatched in the dark, thus eliminating practically the possibility of degradation by light. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish conditions for storage (long-term stability) as well as conditions for dispatch to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C could be reached and stability under these conditions must be demonstrated, if transport without cooling will be applied.

The ERM-BF436e material was selected for the short-term stability study because it is a mixture of both GM and non-GM base material and allows to assess the stability of each base material. Moreover, it is the mixture with the highest GM mass fraction, enabling the best method intermediate precision ($s_{wb,rel}$) of all three mixtures (Table 3).The short-term stability study was carried out using an isochronous design [14]. In this approach, samples of ERM-BF436e were stored for a certain time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples are analysed simultaneously under intermediate precision conditions. Analysis of the material (after various exposure times and temperatures) under intermediate precision conditions greatly improves the sensitivity of the stability tests.

ERM-BF436 is a dried soya powder, which has been processed similarly to other GMO CRM soya powders. As the water content of these powders and their particle size are similar, stability data obtained in the frame of the stability monitoring of soya GMO CRMs were used for the estimation of the uncertainty instead of an individual long-term stability study.

5.1 Short-term stability study

For the short-term stability study, units of ERM-BF436e were stored at 4 °C, 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Five units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured by real-time PCR. The measurements were performed under intermediate precision conditions with respect to the PCR plates, and in a randomised sequence to be able to separate a potential analytical drift from a trend over storage time.

The obtained data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. No statistical outliers were detected for the analyte, and the results were retained for the estimation of u_{sts}.

Furthermore, the data were evaluated against storage time and regression lines of mass fraction versus time were calculated, to test for potential increase/decrease of the DAS-44406-6 soya mass fraction due to shipping conditions. The slopes of the regression lines were tested for statistical significance. None of the trends was statistically significant on a 99 % confidence level for any of the temperatures.

The material can be dispatched without further precautions under ambient conditions.

The results of the measurements are shown in Annex C.

5.2 Long-term stability study

Data from the post-certification monitoring program for GMO CRMs were available. Previously released soya powder CRMs were measured at 20 occasions over a period of more than 9 years. At each occasion, measurements were performed simultaneously on one PCR plate on units stored at 4 °C and -70 °C. In fact, each of these studies can be seen as a two-point isochronous study. The evaluation is based on the ratio of samples from 4 °C and -70 °C.

To verify that the data obtained from stability monitoring can be used to estimate the stability uncertainty contribution for ERM-BF436, an additional isochronous study was organised within the frame of the short-term stability assessment (Section 5.1). The data of the 4 °C short-term stability study did not contradict with the data obtained from the stability monitoring.

The obtained data were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. No statistical outliers were detected, and the results were retained for the estimation of u_{lts}.

Furthermore, the data were evaluated against storage time and regression lines were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected on a 99 % confidence level.

The material can therefore be stored at 4 °C.

The results of the long-term stability measurements are shown in Annex D.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can rule out degradation of materials completely, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method intermediate precision, i.e. to estimate the uncertainty of stability. This means, even under ideal conditions, the outcome of a stability study can only be "degradation is 0 ± x % per time".

Uncertainties of stability during dispatch and storage were estimated as described in [15]. For this approach, the uncertainty of the linear regression line with a slope of zero is calculated. The uncertainty contributions u_{sts} and u_{lts} are calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot t_{tt} \qquad \textbf{Equation 4}$$

$$u_{lts,rel} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot t_{sl} \qquad \textbf{Equation 5}$$

RSD relative standard deviation of all results of the stability study

x_i result at time point *i*

$\bar{x}$ mean results for all time points

t_{tt} chosen transport time (1 week at 60 ºC)

t_{sl} chosen shelf life (24 months at 4 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 60 °C studies. The uncertainty describes the possible change during a dispatch at 60 °C lasting for 1 week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the stability monitoring program for soya GMO CRMs. The uncertainty contribution describes the possible degradation during 24 months storage at 4 °C.

The results of these evaluations are summarised in Table 4.

Table 4: Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of 60 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of 4 °C and 24 months.

Candidate CRM	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-BF436	1.2	1.8

After the certification campaign, the material will be subjected to IRMM's regular stability monitoring programme to control their further stability.

6 Characterisation by gravimetric preparation

The material characterisation is the process of determining the property value of a reference material.

The material characterisation was based on a primary method of measurement confirmed by independent analysis. Gravimetric mixing was chosen as the method of choice. The five candidate CRMs under the label ERM-BF436 are soya powder materials processed from non-GM and GM seeds. While ERM-BF436a is prepared from the pure blank material and ERM-BF436b from the pure GM material, the other candidate CRMs of the ERM-BF436 series are gravimetrically produced mixtures of the pure non-GM and GM seed powders. ERM-BF436 is certified for the mass fraction of DAS-44406-6.

6.1 Purity of the base materials

The purity of the GM and non-GM batches used for the processing of these powders was investigated to be able to calculate the certified value. No indication was found that the GM DAS-44406-6 soya material contained seeds being negative for the event DAS-44406-6 (Section 3.1). No indication for the presence of DAS-44406-6 was found in the non-GM powder by real-time PCR (Sections 3.1 and 3.5). As no evidence for a contamination was found in both base materials, 100 % purity was used for the calculation of the certified mass fraction of DAS-44406-6 in the powder mixtures. The difference between the statistically established purity of 98.6 % (Section 3.1) and the 100 % purity is taken into account in the uncertainty calculation.

The powder used for the production of ERM-BF436a did not contain traces of DAS-44406-6 above the LOD of the applied real-time PCR method (Sections 3.1 and 3.5). The certified value for ERM-BF436a is therefore based on the LOD of the real-time PCR method applied, as determined during in-house method validation.

6.2 Mass fractions and their uncertainties

The mass values are based on the mass fractions of dry-mixed GM and non-GM powder, corrected for their water mass fractions and taking into account the powder's purity with regard to the DAS-44406-6 event. The values were calculated according to the following equations:

$$\text{GM mass fraction [g/kg]} = \frac{m_{\text{GM,anhyd}}\,[\text{g}] \times p_{\text{GM}}\,[\text{g/g}]}{m_{\text{GM,anhyd}}\,[\text{g}] + m_{\text{nonGM,anhyd}}\,[\text{g}]} \times 1000 \qquad \textbf{Equation 6}$$

$$m_{\text{GM,anhyd}}\,[\text{g}] = m_{\text{GM}}\,[\text{g}] \times (1 - \text{WMF}_{\text{GM}}\,[\text{g/g}]) \qquad \textbf{Equation 7}$$

$$m_{\text{nonGM,anhyd}}\,[\text{g}] = m_{\text{nonGM}}\,[\text{g}] \times (1 - \text{WMF}_{\text{nonGM}}\,[\text{g/g}]) \qquad \textbf{Equation 8}$$

(anhyd = anhydrous; p_{GM} = purity of the GM powder used for the dilution; WMF = water mass fraction)

In Table 5, the data supporting the calculation of the mass fractions of DAS-44406-6 soya are summarised.

Table 5: Subsequent mixing of GM DAS-44406-6 soya seed powder (ERM-BF436b) with non-GM powder (ERM-BF436a) to prepare the ERM-BF436c, d and e materials

Candidate CRM	GM powder			Non-GM powder [1)]	Mixtures
	Mass fraction of GM powder [g/kg]	Water mass fraction $\pm$ U (k = 2) [g/kg]	Mass [g]	Mass [g]	Resulting mass fraction of GM powder [g/kg]
ERM-BF436e	1000.0	9.3 $\pm$ 1.3	400.0	3599.8	100.0
ERM-BF436d	100.0 [2)]	11.5 $\pm$ 1.6	400.8	3599.0	10.0
ERM-BF436c	10.0 [3)]	11.1 $\pm$ 1.6	400.6	3599.2	1.0

[1)] The non-GM powder (ERM-BF436a) used for the gravimetric preparations had a water mass fraction of 9.0 $\pm$ 1.3 g/kg (U, k = 2) and was considered to be free of DAS-44406-6 soya.

[2)] For the preparation of ERM-BF436d the ERM-BF436e GM powder mixture was used.

[3)] For the preparation of ERM-BF436c the ERM-BF436d GM powder mixture was used.

The uncertainties on the certified mass fractions (u_{char}) of DAS-44406-6 soya are composed of several contributions, i.e. the uncertainty on the mass determination ($u_{char,1}$), the uncertainty on the water mass fraction analysis ($u_{char,2}$), and the uncertainties on the purity determination of the non-GM and GM base powders ($u_{char,3}$ and $u_{char,4}$). Based on a statistical analysis of the probability distribution to find a negative seed in the GM base material, it could be concluded that the purity was higher than 98.6 % (95 % confidence level, Section 3.1). This value was taken into account when estimating the uncertainty of the certified value (Table 6).

Table 6: Uncertainty budgets for the mass fractions of DAS-44406-6 soya in ERM-BF436

Candidate CRM	Nominal mass fraction [g/kg]	Standard uncertainty contribution [g/kg]				Combined uncertainty u_{char} [g/kg]
		$u_{char,1}$ [1)]	$u_{char,2}$ [2)]	$u_{char,3}$ [3)]	$u_{char,4}$ [4)]	
ERM-BF436a	0	n.a.[5)]	n.a.[5)]	0.0173	n.a.[5)]	0.0173
ERM-BF436b	1000	n.a.[5)]	n.a.[5)]	n.a.[5)]	3.9479	3.9479
ERM-BF436c	1	0.0016	0.0007	0.0173	0.0039	0.0178
ERM-BF436d	10	0.0128	0.0065	0.0173	0.0395	0.0454
ERM-BF436e	100	0.0905	0.0528	0.0173	0.3947	0.4087

[1)] Standard uncertainty of the mass determination, mainly based on the uncertainty of the balance and the number of weighing steps required.

[2)] Standard uncertainty of the water mass fraction determination by V-KFT.

[3)] Standard uncertainty of the purity estimation of the non-GM base material (LOD = 0.06 g/kg), based on the half-width of the interval between 0 and 0.06 g/kg, divided by the square root of 3 (rectangular distribution).

[4)] Standard uncertainty of the purity estimation of the GM base material (> 98.6 %), based on the interval between 98.6 % and 100 % divided by the square root of 3 (rectangular distribution).

[5)] n.a.: not applicable

6.3 Verification measurements

Real-time PCR measurements demonstrated that no mixing errors were made (Section 3.5). Gel electrophoresis proved that the DNA analyte was not degraded during processing of the candidate CRMs (Section 3.4).

7 Value Assignment

Certified values were assigned. Certified values are values that fulfil the highest standards of accuracy. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

The certified values are based on the masses of dried powder of GM seeds and non-genetically modified seeds used in the gravimetrical preparation. The masses of the powders were corrected for their respective water mass fractions during the preparation of the materials (Table 5).

The assigned uncertainty consists of uncertainties related to characterisation, u_{char} (Section 6.2), potential between-unit inhomogeneity, u_{bb} (Section 4.1), and potential degradation during transport, u_{sts}, and long-term storage, u_{lts} (Section 5). These different contributions were combined to estimate the expanded, relative uncertainty of the certified value ($U_{CRM,rel}$) with a coverage factor k as:

$$U_{CRM,rel} = k \cdot \sqrt{u^2_{char,rel} + u^2_{bb,rel} + u^2_{sts,rel} + u^2_{lts,rel}} \qquad \textbf{Equation 9}$$

- u_{char} was estimated as described in Section 6.2.
- u_{bb} was estimated as described in Section 4.1.

- u_{sts} was estimated as described in Section 5.3.
- u_{lts} was estimated as described in Section 5.3.

For the blank material, the LOD of the method was used to describe the 95 % confidence interval on the certified mass fraction of the event (< 0.06 g/kg). This is supported by the high purity of the (non-GM) material and the absence of any mixing step; calculating the U_{CRM} for the blank material on the basis of the only quantifiable standard uncertainty ($u_{char,3}$) would result in a value of U = 0.04 g/kg (assuming k = 2), which is below the certified < 0.06 g/kg value. The LOD is, therefore, a conservative estimate of the certified value and its uncertainty.

For the pure GM material, the statistically calculated purity of the GM seed batch (Section 3.1) was used to describe the 95 % confidence interval on the certified mass fraction of the event (> 986 g/kg). Calculating U_{CRM} for the pure GM material on the basis of the only quantifiable standard uncertainty ($u_{char,4}$) would result in a value of U = 8 g/kg (assuming k = 2), which is less than the difference between the nominal value (1000 g/kg) and the certified value (> 986 g/kg). The statistically calculated purity is, therefore, a conservative estimate of the certified value and its uncertainty.

A coverage factor k of 2 was applied, to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Table 7.

Table 7: Certified values and their uncertainties for ERM-BF436

Candidate CRM	Certified value [g/kg]	u_{char} [g/kg]	u_{bb} [g/kg]	u_{sts} [g/kg]	u_{lts} [g/kg]	U_{CRM} [3)] [g/kg]
BF436a	< 0.06 [1)]	0.0173	n.a.[4)]	n.a.[4)]	n.a.[4)]	-
BF436b	> 986 [2)]	3.9479	n.a.[4)]	n.a.[4)]	n.a.[4)]	-
BF436c	1.00	0.0178	0.0639	0.0120	0.0180	0.14
BF436d	10.0	0.0454	0.4198	0.1199	0.1799	1.0
BF436e	100	0.4087	3.6992	1.1997	1.7996	9

[1)] With a 95 % confidence, the certified value is below this level.

[2)] With a 95 % confidence, the certified value is above this level.

[3)] Expanded (k = 2) and rounded uncertainty

[4)] n.a.: not applicable

As no proof could be delivered how the certified GM powder mass fractions are related to the corresponding transgenic and soya-specific DNA copy number ratios, the user is reminded that IRMM only certifies these materials for their mass fraction of DAS-44406-6. Additionally, one has to be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements on unknown samples as DNA- and/or protein-based quantification of GMOs may vary with the particular matrix and the soya variety tested.

8 Metrological traceability and commutability

8.1 Metrological traceability

The traceability chain is based on the use of calibrated balances and a thorough control of the weighing procedure. The value is therefore traceable to the SI.

8.2 Commutability

Many measurement procedures include one or more steps, which are selecting specific (or specific groups of) analytes from the sample for the subsequent steps of the whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions expressing this concept. For instance, the CSLI Guideline C-53A [16] recommends the use of the following definition for the term *commutability*:

> "The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and, thus, is a crucial characteristic in case of the application of different measurement methods. When commutability of a CRM is not established, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant.

The candidate CRM is prepared from non-GM and GM soya seed powder and the analytical behaviour will be the same as for a routine sample of milled soya seeds/grains. For other types of samples the commutability has to be assessed.

9 Instructions for use

9.1 Safety and protection of the environment

The usual laboratory safety measures apply. The material is for in-vitro use only; it does not contain any viable seeds.

9.2 Storage conditions

The materials shall be stored at (4 ± 3) °C in the dark. Care shall be taken to avoid change of the moisture content once the units are open, as the material is hygroscopic. The user is reminded to close bottles immediately after taking a sample.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened bottles.

9.3 Minimum sample intake

The minimum sample intake is 200 mg.

ERM-BF436a and ERM-BF436b are pure non-GM and GM materials. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity (Annex E).

9.4 Use of the certified value

The main purpose of these materials is the use for calibration or quality control of DAS-44406-6 soya detection methods. As any reference material, they can be used for establishing control charts or validation studies.

Use as a calibrant

If this matrix material is used as calibrant, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty. Furthermore, it should be noted that using the same material for calibration and quality control limits the control possibilities, as calibrant and quality control material are based on the same raw materials. If unavoidable, it is recommended to use different concentration levels of ERM-BF436 for calibration and for quality control.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [17]).

For assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is described here in brief:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_{meas} \leq U_{\Delta}$ no significant difference between the measurement result and the certified value, at a confidence level of about 95 % exists.

Use in quality control charts

The materials can be used for quality control charts. All CRM-units will give the same result as inhomogeneity was included in the uncertainties of the certified values.

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Annexes

Annex A: CTAB DNA extraction method (in-house modified)

Solutions and reagents

1) CTAB buffer A
   - 1 % (w/v) CTAB
   - 0.7 M NaCl
   - 0.1 M Tris-HCl, pH 8.0
   - 15 mM Na_2EDTA pH 8.0
2) CTAB buffer B
   - 1 % (w/v) CTAB
   - 0.1 M Tris-HCl, pH 8.0
   - 15 mM Na_2EDTA, pH 8.0
3) Chloroform:1-Octanol (24:1)
4) 1.2 M NaCl
5) 2-Propanol
6) 70 % Ethanol
7) TE low buffer
   - 1 mM Tris, pH 8.0
   - 0.01 mM Na_2EDTA, pH 8.0
8) Proteinase K, 20 mg/mL
9) RNase A, 100 mg/mL
10) 2-Mercaptoethanol
11) QIAGEN Genomic-tip 20/G columns (Qiagen, Venlo, NL)
12) Genomic DNA Buffer Set (including G2, QBT, QC and QF) (Qiagen, Venlo, NL)

Protocol

a) Weigh 200 mg powder in a 2 mL microcentrifuge tube.
b) Add 1.3 mL of CTAB buffer A + 5 µL RNase A + 6.5 µL proteinase K + 26 µL 2-mercaptoethanol and mix by vortexing.
c) Incubate 1 h at 65 °C, shaking at 1,400 rpm.
d) Centrifuge 10 min at 16000 x *g* at RT.
e) Transfer 750 µL of supernatant to a 2 mL microcentrifuge tube containing 1 mL of chloroform:1-octanol (24:1).
f) Mix thoroughly by inverting, incubate 5 min at RT.
g) Centrifuge 10 min at 16000 x *g* at RT.

h) Transfer 600 µL of supernatant to a new 2 mL microcentrifuge tube containing 700 µL of CTAB buffer B.

i) Mix thoroughly by inverting, incubate 30 min at RT.

j) Centrifuge 20 min at 16000 x *g* at RT.

k) Discard the supernatant by pipetting and conserve the pellet.

l) Add 200 µL of 1.2 M NaCl.

m) Incubate 5 min at 50 °C, shaking at 1400 rpm.

n) Add 1.6 mL of G2 buffer + 2.5 µL of RNase A + 20 µL of proteinase K.

o) Incubate 1 h at 50 °C, shaking at 500 rpm.

p) Centrifuge 5 min at 16000 x *g* at RT.

q) Equilibrate a Qiagen Genomic-tip 20/G column with 1 mL of QBT buffer.

r) Apply the sample to the equilibrated Genomic-tip 20/G column.

s) Wash the genomic-tip 20/G column with 3 mL of QC buffer.

t) Elute the genomic DNA with 1 mL of QF buffer (pre-warmed at 50 °C) and collect the DNA in a 2 mL tube.

u) Add 700 µL of 2-propanol to each tube, invert 10 times.

v) Centrifuge 30 min at 10000 x *g* at 4 °C, discard the supernatant by pipetting.

w) Wash the pellet with 1 mL of 70 % ethanol.

x) Centrifuge 10 min at 13000 x *g* at 4 °C.

y) Discard the supernatant by pipetting and air-dry the pellet for about 10 min.

z) Dissolve the DNA pellet in 80 µL of TE low buffer preheated at 50 °C.

a1) Incubate 10 min at 50 °C, shaking at 500 rpm.

a2) Let the pellet dissolve completely overnight at RT.

a3) Store at +4 °C (short term) or -20 °C (long term).

Annex B: Results of the homogeneity measurements

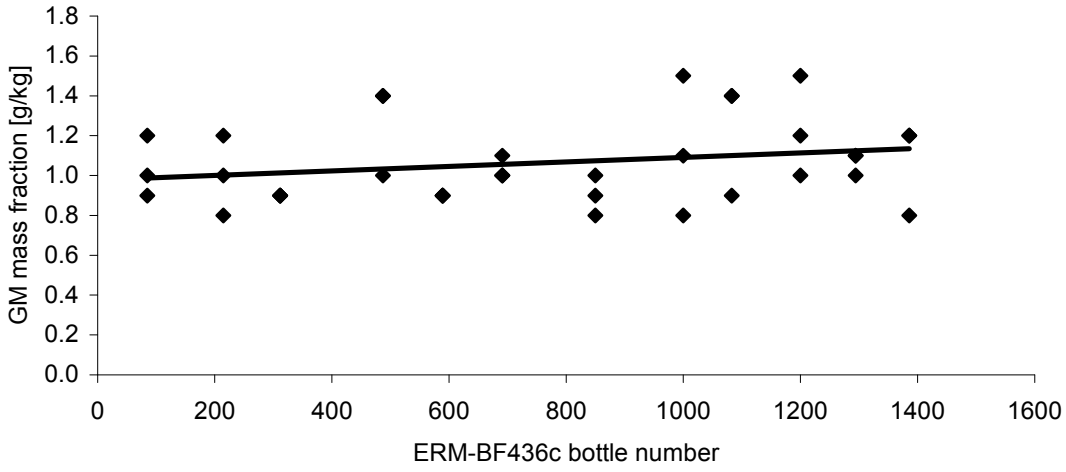

Figure B1: Real-time PCR measurement results obtained for ERM-BF436c. Three samples (extraction replicates) were measured from each 12 random selected bottles (*N* = 12, *n* = 3), with each sample measured in 3 real-time PCR replicates. The linear regression for all data points is given.

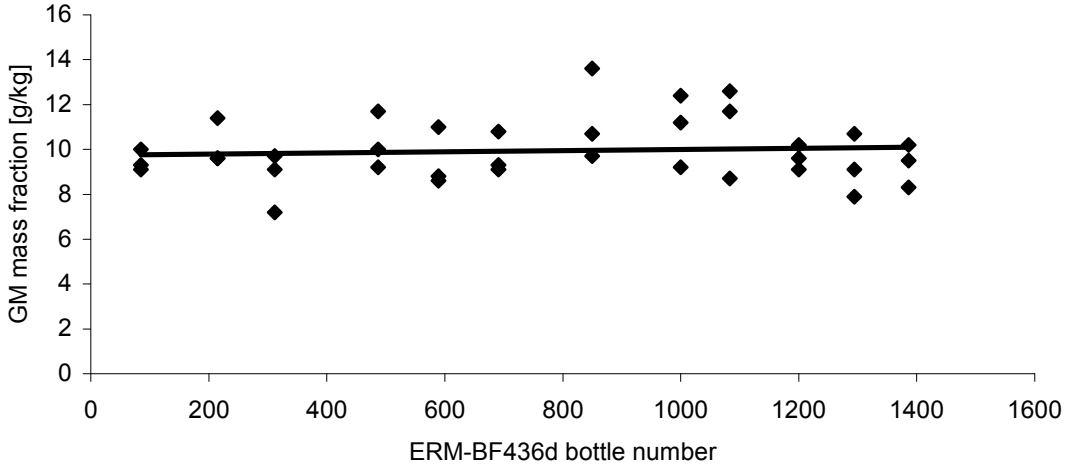

Figure B2: Real-time PCR measurement results obtained for ERM-BF436d. Three samples (extraction replicates) were measured from each 12 random selected bottles (*N* = 12, *n* = 3), with each sample measured in 3 real-time PCR replicates. The linear regression for all data points is given.

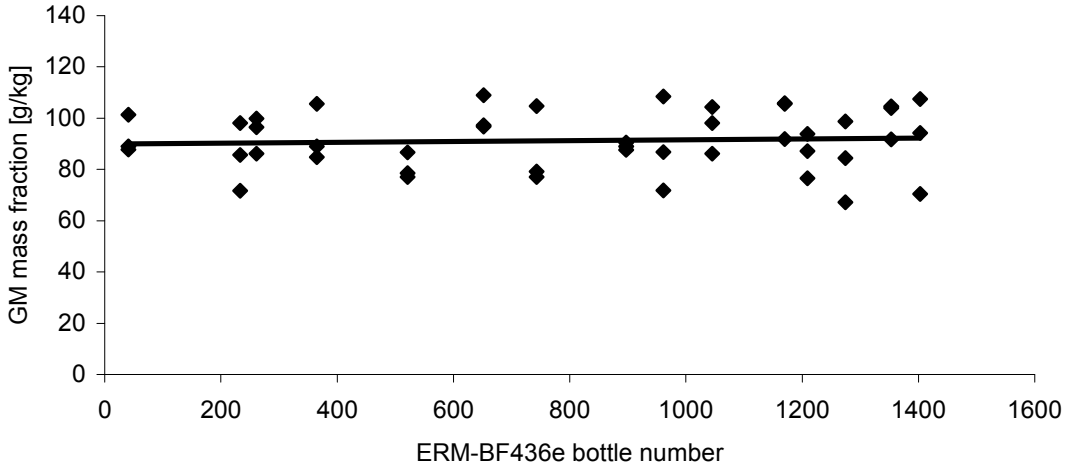

Figure B3: Real-time PCR measurement results obtained for ERM-BF436e. Three samples (extraction replicates) were measured from each 15 random selected bottles (*N* = 15, *n* = 3), with each sample measured in 3 real-time PCR replicates. The linear regression for all data points is given.

Annex C: Results of the short-term stability measurements

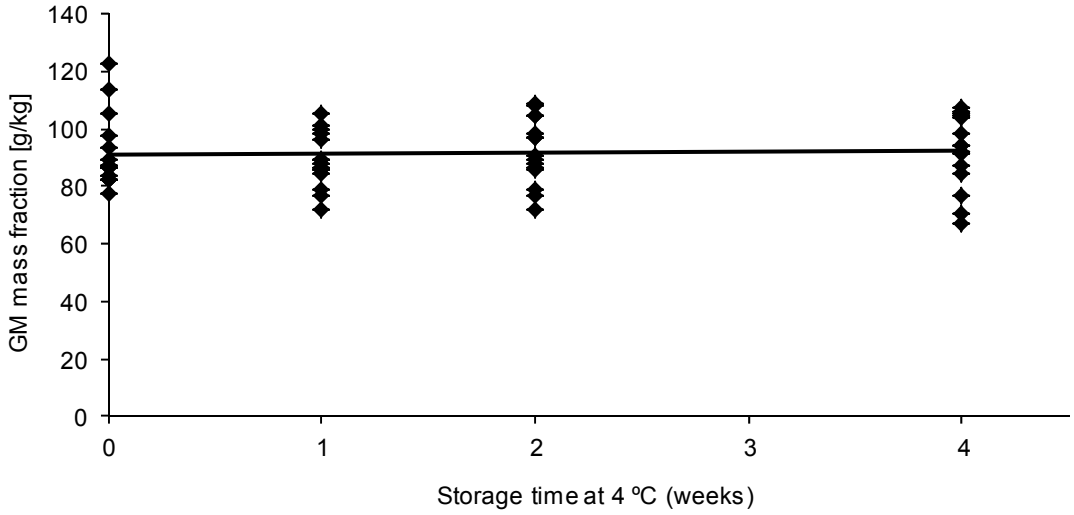

Figure C1: Real-time PCR measurement results obtained for ERM-BF436e during short-term stability testing at 4 °C. For each storage time, 3 samples (extraction replicates) were measured from each 5 random selected bottles (N = 5, n = 3), with each sample measured in 3 real-time PCR replicates. The linear regression for all data points is given.

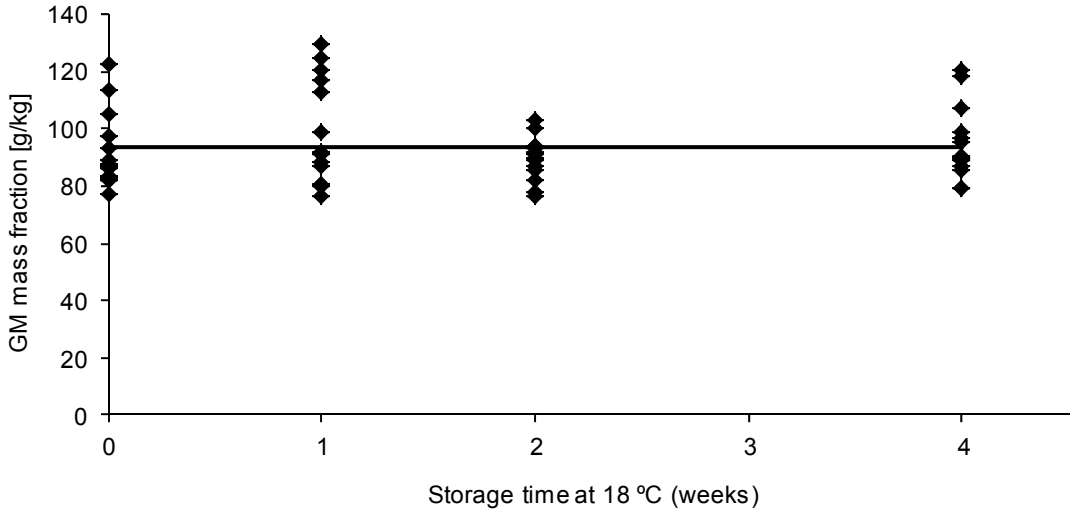

Figure C2: Real-time PCR measurement results obtained for ERM-BF436e during short-term stability testing at 18 °C. For each storage time, 3 samples (extraction replicates) were measured from each 5 random selected bottles (N = 5, n = 3), with each sample measured in 3 real-time PCR replicates. The linear regression for all data points is given.

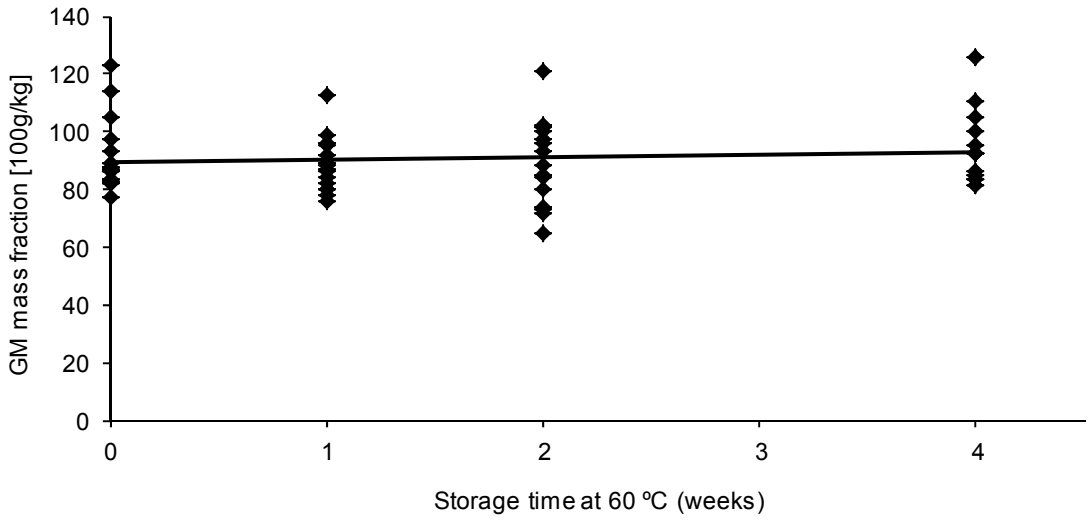

Figure C3: Real-time PCR measurement results obtained for ERM-BF436e during short-term stability testing at 60 °C. For each storage time, 3 samples (extraction replicates) were measured from each 5 random selected bottles (N = 5, n = 3), with each sample measured in 3 real-time PCR replicates. The linear regression for all data points is given.

Annex D: Results of the long-term stability measurements

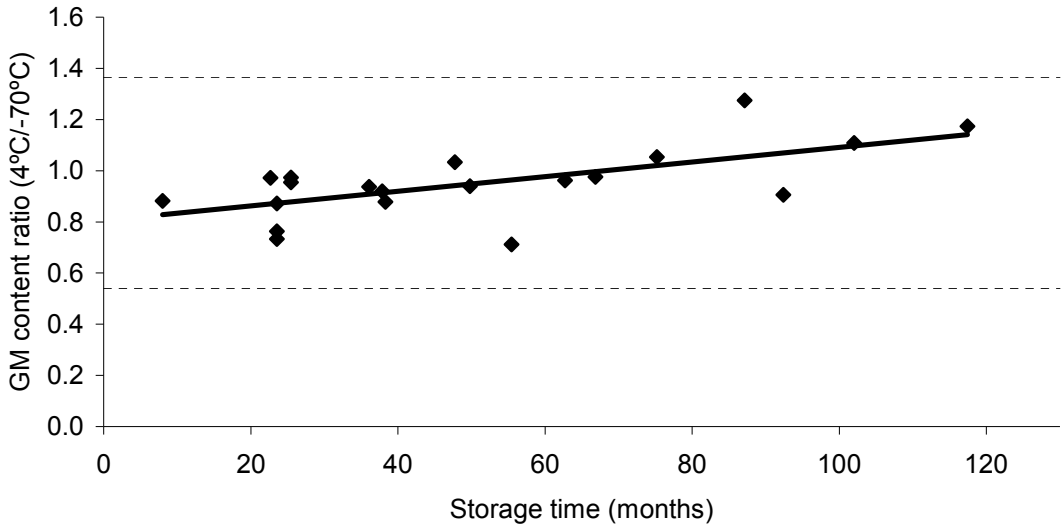

Figure D1: Real-time PCR measurement results obtained for ERM-BF410dk, ERM-BF410e, ERM-BF410S, ERM-BF425c and ERM-BF426c during post-certification monitoring. The dashed lines give the limits of 3 *s* obtained for the measurement results. The linear regression for all data points is given.

Annex E: Preparation of a powder mixture with smaller sample sizes than the minimum sample intake

If no CRM with the required GM concentration level is available, a mixture may be produced by the laboratory using available pure non-GM and GM CRMs certified for their purity.

The required sample needs to be weighed at mass level of the powders using an analytical balance. If both powders are weighed and a co-extraction procedure [18] is applied, there is no need for a mixing step at powder level. As such, the minimum sample intake has not to be respected for each component individually, but for the combined sample.

In order to avoid the introduction of a bias due to different water contents of the GM and non GM material, it is recommended to equilibrate the water content of both powders for 24 h (spread the powder on a dish and expose to the air in the laboratory).

Attention has to be paid that the laboratory also estimates the standard uncertainty for the sample produced. Four standard uncertainty sources have to be considered:

- uncertainty arising from the purity of the non-GM material,
- uncertainty arising from the purity of the GM material,
- uncertainty arising from the weighing of the non-GM material and
- uncertainty arising from the weighing of the GM material.

The uncertainty of the GM and non-GM material purity is taken from the certificate. The relative standard uncertainty of the used analytical balance contributes to the combined standard uncertainty during each weighing step. The combined uncertainty is calculated by taking the square root of the sum of the individual standard uncertainties:

$$u = \sqrt{u_{p(1)}^2 + u_{p(2)}^2 + u_{w(1)}^2 + u_{w(2)}^2}$$

u	combined relative standard uncertainty
$u_{p(1)}$	relative standard uncertainty related to the purity of the non-GM material
$u_{p(2)}$	relative standard uncertainty related to the purity of the GM material
$u_{w(1)}$	relative standard uncertainty related to the weighing of the non-GM material
$u_{w(2)}$	relative standard uncertainty related to the weighing of the GM material

The combined standard uncertainty is calculated. If the expanded uncertainty is required, the obtained standard uncertainty has to be multiplied with the appropriate coverage factor [4].

The here described approach is further outlined in [18].

European Commission

EUR 25997 EN – Joint Research Centre – Institute for Reference Materials and Measurements
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ERM®-BF436a, ERM®-BF436b, ERM®-BF436c,ERM®-BF436d and ERM®-BF436e
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Abstract
This report describes the production of a set of Certified Reference Materials (CRMs) ERM BF436a, b, c, d and e, certified for their DAS-44406-6 mass fractions. The material was produced following ISO Guide 34:2009.
Genetically modified (GM) seeds of the soya event DAS-44406-6 and of a non-GM soya variety were milled to obtain GM and non-GM powders. Gravimetric mixtures of non-GM and GM soya powder were prepared by dry-mixing.
Between-unit homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006.
The certified value was obtained from the gravimetric preparations, taking into account the purity of the base materials and their water mass fraction. The certified values were confirmed by event-specific real-time PCR as independent verification method (measurements within the scope of accreditation to ISO/IEC 17025:2005).
Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties related to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).
The materials are intended for the calibration or quality control of methods. As any reference material, they can be used for establishing control charts or validation studies. The CRMs are available in glass vials containing at least 1 g of dried soya seed powder and closed under argon atmosphere. The minimum amount of sample to be used is 200 mg.
The CRMs were accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

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